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Human papillomavirus-16 associated squamous cell carcinoma of the head and neck (SCCHN): A natural disease model provides insights into viral carcinogenesis

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Abstract

Uncertainty regarding the causality of human papillomaviruses (HPVs) in squamous cell carcinoma of the head and neck (SCCHN) necessitates better *in vitro* models. We carried out molecular analyses of a novel, naturally HPV-16-transformed SCCHN cell line (UPCI:SCC090) and show high copy number of HPV-16 DNA, present in a head to tail, tandemly repeated integrated state. Sequence analysis of the HPV-16 long control region (LCR) in UPCI:SCC090 revealed a deletion of 163 bp, removing a portion of the enhancer sequence, including the binding sites for the transcription factors YY1 and NF1. The E6 and E7 oncogenes of HPV-16 are expressed at high levels in this cell lines, as determined by quantitative reverse transcriptase-polymerase chain reaction (RT-PCR). UPCI:SCC090 contains wild-type tumour suppressor *TP53* gene, and undetectable p53 protein, except after treatment with cisplatin, specific proteasome inhibitors or by E6 RNA interference, suggesting E6-dependent degradation of p53 in this cell line. The results of our studies are consistent with a causative role of HPV-16 in the pathogenesis of SCCHN.

Keywords: Human papillomavirus; Head and neck cancer

1. Introduction

Human papillomaviruses (HPVs) are small, double-stranded DNA viruses that are associated with upper and lower genital tract neoplasias [1,2]. HPV types 16 and 18 are associated with a majority of cases of HPV-induced cervical cancers [3–5]. In most benign and preneoplastic cervical lesions, the HPV DNA is present as a nuclear plasmid. However, in carcinomas the HPV DNA is usually found to be integrated into one or more human chromosomes [6], leading to upregulation of the viral E6 and E7 genes and tumour progression. Although there has been an increase in the number of studies dealing with the role of HPVs in squamous cell carcinoma of the head and neck (SCCHN) during carcinogenesis, such studies have been hampered by the inability to study the disease *in vitro*. Such studies require a well-characterised cellular model of HPV-associated SCCHN for molecular analyses similar to those used in the study of cervical carcinomas [7].

There were approximately 500 000 new cases of SCCHN worldwide in 2001 [8], usually associated with such risk factors as heavy consumption of alcohol and/

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or tobacco. Although the distribution of episomal and integrated HPV forms in both precancerous and cancerous lesions of the head and neck has not been determined, limited evidence suggests similar mechanisms to those observed in cervical carcinomas [9–13]. Epidemiological and molecular studies have shown that more than 90% of cervical tumours exclusively harbour integrated viral sequences [14]. Molecular studies of HPV-associated SCCHN are necessary for a better understanding of the physical state and potential role of this virus in carcinogenesis, and for the development of new, more targeted therapeutic strategies.

The carcinogenic mechanism of HPV-induced SCCHN may differ from that of anogenital cancers. Although well-characterised *in vitro* cellular model systems exist for cervical carcinoma, including the CaSki, HeLa and SiHa cell lines, no clearly defined model for HPV-associated SCCHN exists, despite occasional reports of HPV DNA in head and neck cancer cell lines. Furthermore, the SCCHN field is complicated by the reporting of oral or gingival keratinocyte cultures that are transiently or stably transfected with genes encoding E6 and E7, without the presence of the other HPV genes [15]. Thus, the ideal *in vitro* model for HPV-associated SCCHN would be a naturally HPV-16-transformed cell line derived from a *de novo* tumour from the oropharynx of a SCCHN patient. Here, we report the molecular characterisation of a recently identified HPV-16$^+$ oropharyngeal cell line (UPCI:SCC090).

2. Materials and methods

2.1. Cell lines

Cell lines were cultured as described in Ref. [16]. SiHa, C-33A, PCI-30, PCI-13 (gifts from Dr. Theresa Whiteside, UPCI), L-18 ([17], a gift from Lou Laimins), and UPCI:SCC090: DMEM + 10% FBS (fetal bovine serum) + 2% L-glutamine + 1% Penicillin/Streptomycin (Invitrogen). For CaSki cells, Roswell Park Memorial Institute (RPMI) 1640 was used.

The UPCI:SCC090 cell line was derived by the explant method from a 44-year old male (now deceased) smoker with an oropharyngeal SCCHN arising in the base of tongue. His tumour was staged as T2N1M0 according to the 4th Edition American Joint Committee on Cancer (AJCC) guidelines, and the histology was moderately to poorly differentiated invasive squamous cell carcinoma with basaloid features.

2.2. HPV-positivity, Southern blotting and the identification of integrated HPV-16 DNA

DNA from the following cell lines was isolated by phenol–chloroform–isoamyl alcohol (25:24:1) extraction and ethanol-precipitation [18]: UPCI:SCC90; the cervical carcinoma cell line, CaSki, containing tandemly integrated copies of HPV-16 DNA; and L-18, a human keratinocyte cell line containing episomal copies of HPV-18 DNA [17]. HPV-positivity in the UPCI:SCC090 cell line was tested using M09/M11 PCR primers which amplify an approximately 450-bp conserved region of the L1 gene of HPVs [19]. HPV-16-specific polymerase chain reaction (PCR) was done by amplifying a 477-bp region of the E6 gene of this virus using 5′-ATGCACCAAAAGAGAACTGC-3′ as the forward primer, and 5′-TTACAGCTGGGTTTCTCTAC-3′ as the reverse primer. The glyceraldehyde-3-phosphate dehydrogenase (G3PDH) gene was amplified as a control using the forward primer 5′-ACCACAGTCCATGCCATCAC-3′ and the reverse primer 5′-TCCACCACCCTGTTGCTGTA-3′ which amplify a 556-bp DNA region. PCR for the L1, E6 and G3PDH were performed in a 50 μl volume containing 50 mM KCl, 10 mM Tris (pH 8.3), 1.5 mM $MgCl_2$, 0.01% gelatin, 200 μM deoxynucleoside triphosphate (dNTP) mix, 0.4 μM of each primer and 2.5 units of the Taq DNA polymerase. The DNA was denatured at 94 °C for 5 min, followed by 40 PCR amplification cycles that consisted of denaturation (94 °C, 1 min), annealing (55–60 °C, 1 min) and extension (72 °C, 2 min). An additional extension step of 72 °C for 5 min was included at the end of the reaction. The PCR-amplified DNA was analysed by agarose gel electrophoresis [20]. For Southern blot analysis, 5 μg of CaSki and UPCI:SCC090 DNA was digested with three different restriction endonucleases. *Bgl*II does not cleave the HPV-16 genome, *Bam*HI cleaves it once and *Kpn*I cleaves it at two sites. For the L-18 cell line, the DNA was treated with *Bgl*II which does not cleave the HPV-18 DNA, *Eco*RV (which cleaves it once) and *Bam*HI that cleaves the HPV-18 DNA twice. The digested DNA was electrophoresed on a 0.7% agarose gel and the Southern blots were probed with a ^{32}P-labelled plasmid containing the complete HPV-16 genome as described in Ref. [18]. The blots were subjected to autoradiography at −80 °C. The identity of HPV-16 in UPCI:SCC90 was confirmed by PCR amplification of portions of the E2, E6, E7 and L1 genes followed by automated DNA sequencing of the amplified products. The following HPV-16-specific primers were used for the PCR amplification. E2 (1,228 bp product) forward primer 5′-GGAAATCCAGTGTATGAGCTTAATG-3′ and reverse primer 5′-GTAATGTTGTGGATGCAGTATCAAG-3′; E6/E7 (735 bp product) forward primer 5′-ATGCACCAAAAGAGAACTGC-3′ and reverse primer 5′-TGCCCATTAACAGGTCTTCC-3′. The MY09/MY11 primers [19] were used for the amplification of the L1 gene and primers described above for the amplification of the E6 region of HPV-16. The PCR conditions were the same as described above, except for

the E2 gene for which extension reactions were carried out for 2.5 min. DNA sequencing was carried out using an Automated Applied Biosystems PRISM 3100 Genetic Analyzer.

2.3. Identification of the region deleted in the HPV-16 DNA in the UPCI:SCC090 cell line

The long control region (LCR) of the HPV-16 DNA was amplified by PCR. The sequences of the primers used were: 5′-TTTTGGCACAAAATGTGTTTTT-3′ for the forward primer (HPV-16 positions 7470–7491) and 5′-GCACAGAGCTG CAAACAACTAT for the downstream primer (positions 150–171). The reaction mixtures contained 200 μM of each deoxynucleoside triphosphate (dNTP), 200 ng of UPCI:SCC090 DNA, 1 μM of each primer, and 5 units of the *Pfu* polymerase (Stratagene, La Jolla, CA). The conditions of amplification were as follows: 94 °C for 5 min; 94 °C for 1 min, 60 °C for 1 min, and 72 °C for 2 min for 40 cycles; and 72 °C for 7 min. The PCR-amplified DNA was isolated by gel electrophoresis and subjected to automated DNA sequencing.

2.4. Quantitative real-time PCR (qPCR)

Relative HPV-16 E6 and E7 DNA copy number in the UPCI:SCC090 cells was determined by type-specific primers/probe and conditions [21]. Control DNA quantification was performed, amplifying a series of microsatellite repeats (QuMA) [22], and using a serially diluted HPV-16 E6-encoding plasmid. Comparison was made with CaSki and SiHa (see Table 1). Input copy numbers were determined using HPV-16 E6-containing plasmid DNA, and unknown samples normalised to E6 input plasmid amounts. Relative expression of E6 and E7 was calculated using the delta CT method described previously in Ref. [22]: (Relative expression = $2^{-\Delta CT}$; where $\Delta CT = C_{T(\text{Target gene})} - C_{T(QuMA)}$). While this equation is exactly accurate only for quantitative real-time RT-PCR (qRT-PCR) reactions that are 100% efficient, it provides an estimate, particularly when compared with internal known reagents, such as the E6-encoding plasmid and the CaSki cell line, which has been quantified in terms of the integrated HPV DNA present.

Table 1
HPV-16 qPCR in SCC90 cells

Cell line	[E6][a]	[E7][a]
SCC90	91	171
CaSki	105	249
SiHa	0.46	1.0

qPCR, quantitative real-time PCR.
[a] Relative to control QuMa DNA amplification, using $2^{-\Delta CT}$ method.

2.5. Isolation of RNA and RT-PCR analysis

Expression of the HPV-16 E2 and E6 genes was investigated by reverse transcriptase (RT)-PCR analysis. RNA was isolated using the ULTRASPEC RNA isolation system (Biotecx) according to the manufacturer's protocol. Before DNA synthesis, RNA was treated with DNaseI, amplification grade (Invitrogen) for 15 min at room temperature to avoid DNA contamination. DNaseI was then inactivated by the addition of 25 mM ethylene diamine tetraacetic acid (EDTA) followed by incubation at 65 °C for 10 min. The lack of contaminating DNA was confirmed by the failure of PCR amplification in reactions containing the Taq polymerase, but lacking the reverse transcriptase. The cDNA synthesis was performed at 37 °C for 1 h in a final volume of 20 μl using 1 μg of total RNA template, 0.5 μg of oligo $(dT)_{15}$, 10 mM dNTPs, 30 U of RNase inhibitor and 200 units of MMLV reverse transcriptase. Expression of the HPV-16 E6 gene was studied using the primer pair described above. To detect expression of the HPV-16 E2 gene, PCR amplification was done using 5′-AAAGTGGACATTACAAGACGTTAGC-3′ for the forward primer and 5′-GTGAGCTGTTAAATG-CAGTGAGG-3′ for the reverse primer that are expected to generate a 554-bp product. The expression of the cellular G3PDH gene was used as a control. PCR was performed in a 50 μl volume containing 50 mM KCl, 10 mM Tris (pH 8.3), 1.5 mM $MgCl_2$, 0.01% gelatin, 200 μM dNTP mix, 0.4 μM of each primer and 2.5 units of the Taq DNA polymerase. The DNA was denatured at 94 °C for 5 min, followed by 40 PCR amplification cycles that consisted of denaturation (94 °C, 1 min), annealing (55–60 °C, 1 min) and extension (72 °C, 2 min). An additional extension step of 72 °C for 5 min was included at the end of the reaction. The PCR products were analysed by electrophoresis on 1% agarose gels.

2.6. Quantitative real-time RT-PCR

Reverse transcription was performed with random hexamer primers and Superscript ∏ (Invitrogen Corp.) as described previously in Ref. [9]. As described in Ref. [21], qRT-PCR was then carried out on the Applied Biosystems 7700 Sequence Detection Instrument at 95 °C for 12 min, PCR was performed at 95 °C for 15 s, 60 °C for 60 s. Relative expression of the target gene: endogenous control gene, β-glucuronidase (GUS), was calculated using the delta CT method described previously: (Relative expression = $2^{-\Delta CT}$; where $\Delta CT = C_{T(\text{Target gene})} - C_{T(GUS)}$) [20].

2.7. RNA interference (siRNA) for HPV-16 E6

UPCI:SCC090 cells were plated in 24-well plates in media, as above. At 50% confluence, cells were transfected with Oligofectamine (Invitrogen) following the manufacturer's protocol. siRNA specific for E6, or irrelevant (scrambled sequence) were ordered pre-duplexed and ready for transfection from Dharmacon. Two HPV-16 E6 primers were mixed together using a total concentration of 60 pmol/well. siRNA target sequences are as follows: E6-1 5′-AAGAGCUGAAACAACUAUAC-3′, E6-2 5′-AACUGCGACGUGAGGUAUAUG-3′, IR-REL 5′-AAGCACACACGUAGACAUUCG-3′. Cells were assayed for HPV16-E6 gene activity 48 h after transfection.

2.8. Determination of p53 expression and stability

Cells were treated with the indicated drugs and concentrations (described below) before lysis using l% Nonidet P-40 (NP-40) and protease inhibitor cocktail (Promega). Cisplatin was used at a final concentration of 40 μM. Lactacystin [23] was used at a final concentration of 50 μM; MG-132 was used at a final concentration of 50 μM. Drug treatments were carried out for 6 h prior to cell lysis, sodium dodecyl sulphate-polyacrylamide gel electrophoresis (SDS-PAGE) electrophoresis and Western blotting. Cellular extracts (5–10 μl total protein) were electrophoresed and immunoblotted using anti-p53 DO-7 mAb (BD Pharmingen) and secondary Ab linked to horse radish peroxidase (HRP) or fluorochromes (Amersham Pharmacia). Equal protein loading was determined using the Pierce bicinchoninic acid (BCA) protein quantitation reagent kit and confirmed by blotting the polyvinylidene fluoride (PVDF) membrane with anti-β-actin Ab (Sigma). The intensity of protein bands was determined by densitometric analysis of the immunoblots.

3. Results

3.1. UPCI:SCC090 cells contain integrated HPV-16 DNA

PCR analysis using the consensus M09/M11 primers for the L1 gene showed the presence of a 450-bp band, indicating the presence of HPV DNA in the UPCI:SCC090 cell line (Fig. 1). DNAs from CaSki (HPV-16-positive) and C-33A (HPV-negative) were used as positive and negative controls, respectively. In addition, the G3PDH gene was amplified as a positive control for all the cell lines. Since HPV-16 is the most common type found in SCCHN, HPV-16-specific PCR reactions were performed using DNA from this cell line as the template. PCR analysis using primers for the E6 gene

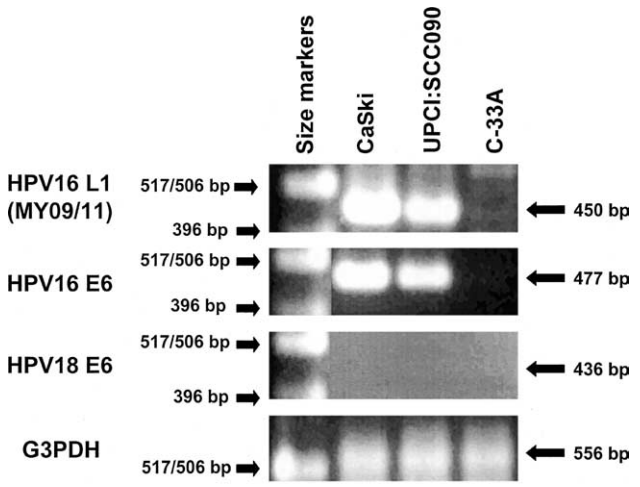

Fig. 1. Polymerase chain reaction (PCR) analysis of UPCI:SCC090 and CaSki DNA. Amplification of human papillomavirus (HPV)-16 genes L1, detected by consensus L1 primers, and E6, amplified using type-specific HPV-16 primers as described in Section 2.

showed that this cell line contained HPV-16 DNA (Fig. 1). Primers specific for HPV-18 E6 gene failed to amplify any DNA from the UPCI:SCC090 cell line (Fig. 1). To confirm that this cell line contains HPV-16 DNA, specific PCR primers were used to amplify portions of the HPV-16 E2, E6, E7 and L1 genes. Automated DNA sequencing of 400–500 bp regions of the above PCR products showed that the DNA corresponded to that of HPV-16 (data not shown). Furthermore, the amplified DNA sequence resembled the European E-G131G variant of HPV-16 [24]. These results confirmed that the UPCI:SCC090 cell line contains HPV-16 DNA.

We then carried out Southern blot analysis to determine the physical state of the HPV-16 DNA in the UPCI:SCC090 cell line. In these studies, we used DNA from CaSki which contains tandem head to tail repeats of integrated HPV-16 DNA [25] and the L-18 cell line that contains episomal HPV-16 DNA [17] as controls. When the Southern blot was hybridised to a HPV-16 probe, uncut UPCI:SCC090 DNA and DNA cleaved with *Bgl*II that does not cleave HPV-16 generated a single, slow-migrating band similar to that observed with the CaSki DNA (Fig. 2). By contrast, uncut L-18 DNA contained two major bands corresponding to the supercoiled (SC) and open-circular (OC) forms of the HPV-18 DNA which cross-hybridises to the HPV-16 probe (Fig. 2). These results demonstrated that as is the case with CaSki cells, the UPCI:SCC090 cell line contains integrated HPV-16 DNA. When the UPCI:SCC090 DNA was treated with *Bam*HI that cleaves the HPV-16 DNA once, a major 7.8 kb band was observed (Fig. 2). Since the HPV-16 DNA is present in an integrated state in this cell line, the 7.8 kb band is presumably generated from the release of unit-length DNA

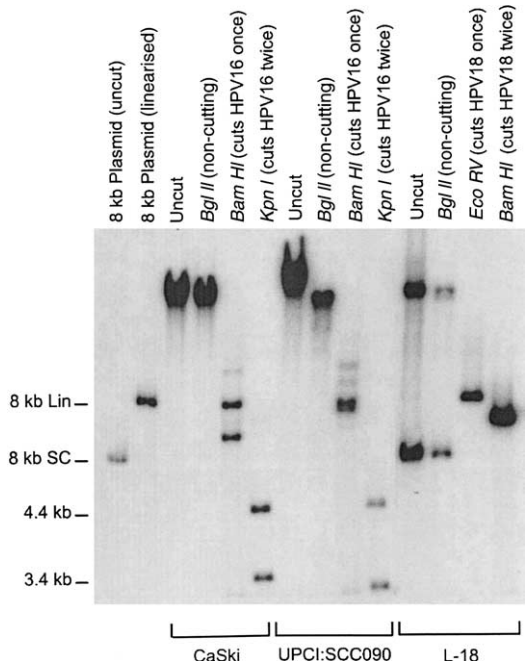

Fig. 2. Physical state of HPV-16 in the UPCI:SCC090 oral carcinoma cell line, as determined by Southern blot analysis (sc, supercoiled).

from tandemly-integrated HPV-16 sequences. CaSki cells that are known to contain only integrated HPV-16 DNA generated two major bands of similar intensity of 7.9 and 6 kb when the DNA from this cell line was treated with *Bam*HI (Fig. 2). The 7.9 kb band corresponds to the unit-length DNA released from tandemly-integrated HPV-16 sequences, while the smaller 6 kb band corresponds to tandemly-integrated HPV-16 DNA containing deletions in the viral genome [25]. Treatment of the UPCI:SCC090 and CaSki DNA with *StuI* and *Nco*I that also cleave the HPV-16 genome once gave results similar to those observed with *Bam*HI (data not shown). As shown previously in Ref. [25], CaSki cells also generated a faint 10.5 kb band that corresponds to HPV-16 DNA containing a duplicated region of the viral genome. Two fainter, larger than unit-length bands observed with the UPCI:SCC090 sample (Fig. 2) may also correspond to integrated HPV-16 genomes containing duplicated viral sequences. Treatment of UPCI:SCC090 and CaSki DNA with *Kpn*I that cleaves HPV-16 twice generated two major bands of 4.4 and 3.4 kb (Fig. 2), consistent with the presence of tandemly integrated HPV DNA. As a control for a cell line containing episomal HPV DNA, the L-18 DNA generated an 7.8-kb band when cleaved with the single cutting *Eco*RV enzyme and fragments of 6.8 and 1 kb (data not shown) upon treatment with *Bam*HI that cleaves the HPV-18 DNA twice (Fig. 2). Taken together, our results suggest that the UPCI:SCC090 cell line contains tandemly repeated copies of HPV-16 DNA integrated into the chromosome.

3.2. Localisation of the region deleted in the HPV-16 LCR

To study the LCR of HPV-16 in the UPCI:SCC090 cell line, we amplified a 606-bp region of the virus by PCR. Agarose gel analysis of the PCR product showed that the reaction product was smaller than the expected size obtained when wild-type HPV-16 DNA present in the W12 cell line [26] was used as the template. To identify the site of deletion, the PCR-amplified LCR DNA from UPCI:SCC090 was subjected to automated DNA sequencing. These results showed that a 163-bp sequence of LCR (nucleotides 7658–7818) was deleted in the HPV-16 genome present in the UPCI:SCC090 cell line.

3.3. HPV-16 genes are transcriptionally active in UPCI:SCC090 cells

RT-PCR analysis was carried out to study the expression of HPV-16 E2 and E6 genes in the UPCI:SCC090 cell line. Before DNA synthesis, RNA was treated with DNaseI, amplification grade (Invitrogen), for 15 min at room temperature to avoid DNA contamination. DNaseI was then inactivated by the addition of 25 mM EDTA followed by incubation at 65 °C for 10 min. As shown in Fig. 3, a single band predicted to be 554 bp in size was obtained with the E2-specific primers. In the presence of E6-specific primers, two expected bands of 296 and 477 bp were observed (Fig. 3), corresponding to products of alternatively-spliced E6 mRNA [27,28]. Thus, UPCI:SCC090 expresses both the regulatory E2

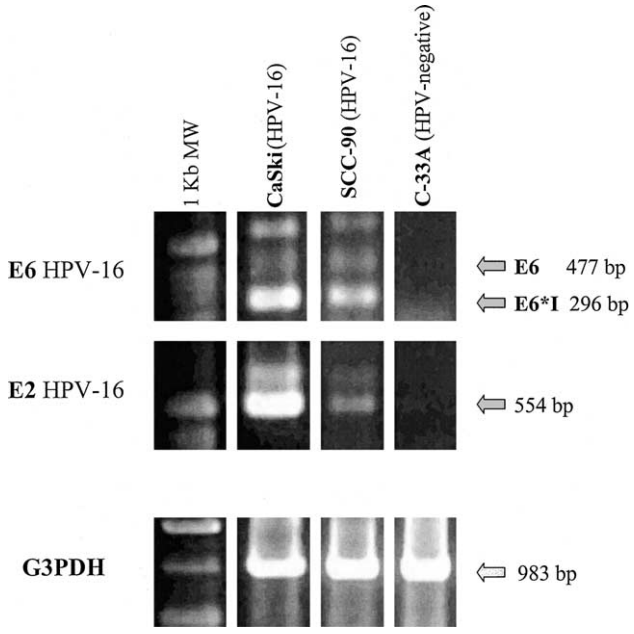

Fig. 3. RT-PCR analysis of HPV-16 E2 and E6 transcripts in UPCI:SCC090 cells.

gene and the E6 oncogene of HPV-16, consistent with the presence of tandemly integrated copies of the virus in this cell line. Further, these results show that integration of the HPV-16 DNA in this cell line does not interrupt the viral E2 gene.

3.4. Quantitative real-time PCR to quantitate relative copies of HPV-16 E6 DNA

We performed qPCR for HPV-16 E6 and E7 gene to quantify viral DNA copy numbers present in UPCI:SCC090 cells, compared with known high (CaSki) and low (SiHa) copy number cervical carcinoma cells. The cycle threshold (Ct) for UPCI:SCC090 cells was *significantly* lower than SiHa cells, which are known to contain 1–2 copies of integrated HPV-16 genomic copies. Back-calculation using quantitatively amplified series of microsatellite repeats as described in Ref. [22], and developing a standard curve based on a serially diluted HPV-16 E6 encoding plasmid, enabled the relative determination of HPV-16 copy numbers in UPCI:SCC090 cells. Thus, UPCI:SCC090 contains approximately 100–150 copies of HPV-16 DNA (Table 1), similar to CaSki cells (known to contain several hundred copies per host cellular genome).

3.5. Real-time quantitative reverse transcription PCR demonstrates high expression of HPV-16 E6 and E7 oncogenes

We measured E6 and E7 mRNA levels in UPCI:SCC090 cells to evaluate the likelihood that HPV-16 contributed to carcinogenesis in these cells. As shown in Fig. 4, high-level E6 and E7 expression was observed. Consistently higher ($P < 0.005$) levels of E6 and E7 expression were observed in UPCI:SCC090 cells compared with CaSki and SiHa cells, indicating the likely importance of these gene products in UPCI:SCC090 carcinogenesis.

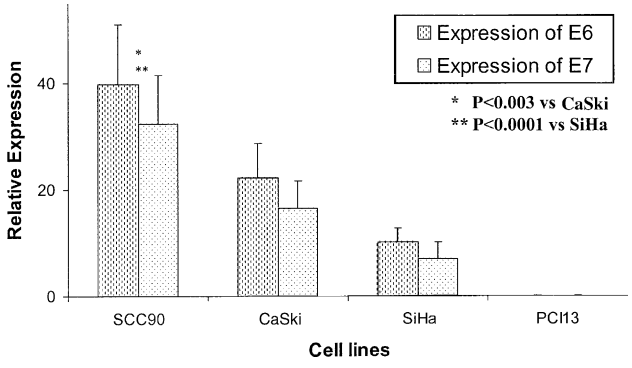

Fig. 4. Expression of HPV-16 E6 and E7 in UPCI:SCC090 cells by quantitative reverse-transcription RT-PCR (qRT-PCR) relative to the control gene, β-glucuronidase (GUS). *Relative to control QuMa DNA amplication, using $2^{-\Delta CT}$ method.

3.6. Genotype of TP53 in UPCI:SCC090 cells

Although *TP53* is mutated in some HPV-16 associated carcinomas, the mutation frequency is lower than in non-HPV-associated tumours, and this correlates with viral gene expression [29]. Consistent with this notion, we performed cDNA sequence analysis for the *TP53* gene in UPCI:SCC090 cells (data not shown). Direct fluorogenic sequence analysis of both coding and non-coding strands, with subsequent BLAST alignment demonstrated that UPCI:SCC090 cells contain wild-type *TP53* in exons 2 through 11. Using differential restriction digestion [30], we confirmed that UPCI:SCC090 cells are heterozygous for proline and arginine at codon 72.

3.7. Reversal of p53 degradation by anti-neoplastic and anti-viral drug treatment

Based on the wild-type sequence of *TP53* in UPCI:SCC090 cells, and E6-induced, ubiquitin-mediated proteolysis of p53, we postulated that antineoplastic chemotherapeutic effects might be mediated through restoration of p53 expression. Fig. 5(a) shows an immunoblot of UPCI:SCC090 lysates, indicating that p53 is essentially undetectable in these cells, likely due to the enhanced ubiquitination and proteasomal degradation induced by HPV-16 E6 protein associating with the E6-AP (ubiquitin E3 ligase). After cisplatin treatment (40 μM for 6 h), reversal of this E6 effect and p53 expression was clearly observed in UPCI:SCC090 and CaSki cells (data not shown). A similar effect was also seen with proteasome inhibitors lactacystin [23] and leucinal–leucinal–norleucinal (referred to as LLnL or MG-132).

3.8. siRNA knockdown of HPV-16 E6 results in accumulation of p53

To clarify the carcinogenic effect of HPV-16 E6 on p53 expression, UPCI:SCC090 cells were transfected with siRNA against HPV-16 E6. E6 RNA levels were reduced up to 70% as shown by qRT-PCR (data not shown). We found that repression of HPV-16 E6 expression led to the accumulation of p53 in these cells (Fig. 5(b)). The level of p53 accumulation observed in siRNA-treated UPCI:SCC090 cells was lower than that seen after treatment with potent proteasome inhibitors (Fig. 5(a)), likely due to incomplete knockdown of E6 expression by siRNA.

4. Discussion

Epidemiological correlation between mucosatropic HPV infection and a subgroup of SCCHN is most com-

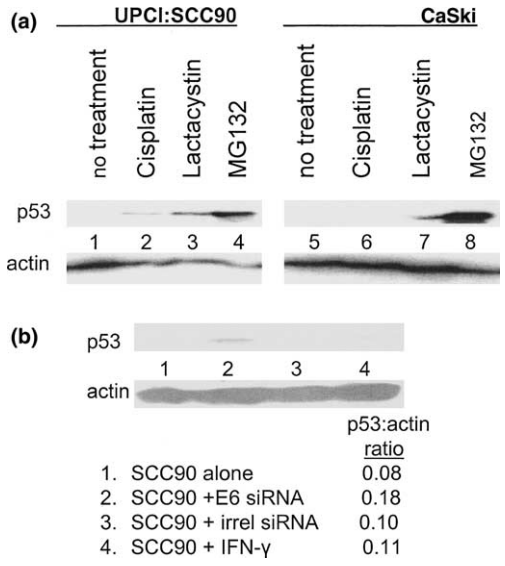

Fig. 5. Recovery of p53 expression by treatment of UPCI:SCC090 cells with the chemotherapeutic agent cisplatin or by proteasome inhibitors (a) or by downregulation of HPV-16 E6 expression by RNA interference (b). (a) Recovery of p53 from HPV-l6-induced degradation. Western blot analysis of the p53 protein in UPCI:SCC090 and CaSki cells using DO-7 anti-p53 monoclonal antibodies after treatment with cisplatin (40 μM for 6 h; lanes 2 and 6) and after treatment with specific proteasome inhibitors lactacystin (10 μM; lanes 3 and 7) or MG-132 (50 μM, lanes 4 and 8). (b) Western blot analysis of the p53 protein after transient transfection of UPCI:SCC090 cells with a 21-mer oligonucleotides designed for short hairpin RNA interference of the E6 gene (siRNA), see Section 2 (1FN-γ, interferon-γ).

pelling for the oropharyngeal site (tonsil or base of tongue). In general, tobacco exposure is inversely associated with the presence of HPV-infection in SCCHN, but these features are not absolute. The determinants of each factor in the aetiology of SCCHN has been difficult to identify due to a lack of natural *in vitro* cellular models of this disease. Cell lines such as we have described in this report are critical to our understanding of this disease and comparing features between HPV-associated SCCHN and cervical carcinoma are limited. Reports of propagation of naturally occurring, HPV-transformed SCCHN cell lines have been infrequent, with few cell lines maintaining their HPV DNA [31,32]. Episomal HPV DNA, by itself, is generally expected to be an unlikely aetiological factor for SCCHN, due to the low-level expression of the transforming E6 and E7 proteins. However, the presence of mutations in HPV LCR could result in an increased expression of these oncogenes [25,31,33–35] and this may lead to cellular transformation. Integrated viral DNA that leads to high levels of E6/E7 expression is much more likely to be present in naturally transformed, HPV-16-infected SCCHN cell lines. Because a causal association of HPV-induced carcinogenesis is uncertain, the need for *in vitro* cellular models is paramount to enable the characterisation of HPV-associated SCCHN.

Recently, it has been shown by fluorescent *in situ* hybridisation (FISH) and restriction-site PCR that the UPCI:SCC090 cell line contains integrated HPV-16 DNA in chromosomes 3, 6, 9q and 13q [33]. Furthermore, the integration sites were found to be generally located in common fragile sites. Our quantitative PCR studies demonstrate that the HPV-16 DNA is present at approximately 100–150 copies per cell in UPCI:SCC090 (Table 1). Southern blot analysis of uncut UPCI:SCC090 DNA with an HPV-specific probe showed the presence of a high molecular weight DNA, but no signal corresponding to the SC or OC forms of HPV-16 DNA was observed (Fig. 2). Since treatment of UPCI:SCC090 DNA with *Bam*HI that cleaves HPV-16 DNA only once generated a single band of 7.8 kb (Fig. 2), our results suggest that the viral genome is integrated in tandem copies in a head to tail orientation in this cell line. Such an arrangement of integrated sequences would release unit length HPV-16 DNA upon treatment with a single-cutting enzyme. This interpretation is consistent with the observations with the CaSki cell line which also generates an 8 kb band upon treatment with *Bam*HI (Fig. 2). However, CaSki cells also generated a second major band of approximately 6 kb, which represents tandem integration of a second, deleted form of HPV-16 DNA in this cell line [25]. Deletions and rearrangements of HPV DNA are frequently observed in cervical carcinoma cell lines containing integrated HPV DNA [33–35]. We used DNA from the L-18 cell line containing episomal copies of HPV-18 DNA as a control. The results obtained with the L-18 DNA were consistent with the presence of episomal HPV-18 DNA in this cell line (Fig. 2).

UPCI:SCC090 cells contain integrated HPV-16 DNA and express high levels of E6, which is important for cellular transformation [36,37]. These observations are consistent with a causative role for HPV-16 in the carcinogenesis of SCCHN. Interestingly, UPCI:SCC090 also expressed the viral E2 gene (Fig. 3). This suggests that, as is the case in CaSki cells, the E2 gene is not disrupted or deleted in the UPCI:SCC090 cell line. Previous studies have identified several mutations in the LCR of HPVs in cases of cervical carcinoma, especially those in which the HPV genome is present in an episomal form [1,34]. PCR amplification of the HPV-16 LCR in the UPCI:SCC090 cell line followed by DNA sequencing showed that a 163-bp sequence (nucleotides 7656–7818) was deleted in the HPV-16 genome present in the UPCI:SCC090 cell line. This region includes a portion of the HPV-16 enhancer sequence, two YY1 binding sites and one NF1 binding site [34,35]. Regions within the HPV LCR are known to negatively regulate HPV-16 E6 and E7 expression. For example, binding of the YY1 factor to its binding site has been suggested to interfere with the formation of the transcription initiation complex at the P_{97} promoter for the E6/E7 genes

[35]. Since the HPV-16 genome in the UPCI:SCC090 cell line contains a deletion of the binding sites for the YY1 and NFI proteins, our results suggest that this may result in the upregulation of E6/E7 expression, even in the presence of viral E2 protein. The additional sequences missing in the HPV-16 LCR in UPCI:SCC090 cells may also play a negative role in E6/E7 oncogene expression. Integrated copies of HPV-16 are also present in the tumour from which the UPCI:SCC090 cell line was derived (data not shown), suggesting that expression of the E6/E7 oncogenes from the integrated state may have contributed to the development of the tumour from which this cell line was derived.

Phenotypic and genotypic analysis shows that UPCI:SCC090 represents an interesting and valid model of HPV-16-associated SCCHN. It is derived from a *de novo* oropharyngeal tumour, a site with roughly 50% prevalence of HPV-16 DNA in numerous studies [38,39]. This cell line contains wild-type p53 which is rapidly degraded in untreated cells, but is recoverable by the action of specific proteasome inhibitors and E6 knockdown by siRNA (Fig. 5), consistent with the functional p53 inactivation by the HPV-16 E6 protein. Biologically active concentrations of cisplatin, a commonly used chemotherapeutic drug in SCCHN, showed similar recovery of p53 expression (Fig. 5(a)). Although cervical cancer may not be as sensitive to this drug as SCCHN, this fact raises the possibility that these diseases although generally associated with the same viral subtypes, may develop through distinct aetiological mechanisms. Such issues may now be tested *in vitro* comparing features of cell lines derived from individuals with each disease.

Because of the mounting molecular epidemiological data in favour of the presence an HPV-16-associated subtype of SCCHN, particularly in the oropharynx, the UPCI:SCC090 cell line promises to be quite valuable as an *in vitro* model system to test similarities between HPV-associated SCCHN, HPV-negative SCCHN, and HPV-induced cervical carcinoma. Further studies are expected to clarify the aetiological role of HPV-16 in a clinically significant subset of SCCHN.

Conflict of interest statement

None declared.

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